Immunology 1998 **95** 314–321

Oxidation of defined antigens allows protein unfolding and increases both proteolytic processing and exposes peptide epitopes which are recognized by specific T cells

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SUMMARY

The participation of oxidative mechanisms in major histocompatibility complex (MHC) class II-restricted antigen presentation was studied *in vitro*. In general, antigen processing is inhibited when peritoneal macrophages (MØ) are incubated with scavengers of reactive oxygen intermediates (ROI): mannitol (an ·OH scavenger), dimethylurea (DMTU, which reacts with H_2O_2 and HOCl) and NCO-700 (an epoxysuccinic acid derivative which inhibits oxidant production by activated phagocytes and can scavenge reactive oxygen species in both NaOCl and hypoxanthine (XOD) systems). However, neither rotenone and antimycins (inhibitors of O_2^- production at the NADH dehydrogenase and ubiquinone–cytochrome b regions, respectively) nor aminoguanidine (an inducible nitric oxide synthase inhibitor) impaired antigen presentation, thus indirectly discarding the participation of mitochondrial oxidation and reactive nitrogen intermediates (RNI) in antigen processing. ROI scavengers do not inhibit the MHC class II-restricted presentation of antigens that need processing but have their disulphide bonds reduced. It can be shown that oxidation of protein antigens (either by chlorination or performic acid treatment) allow protein unfolding and enhance both processing and exposure of immunogenic epitopes to specific T cells.

INTRODUCTION

Although considerable work has been carried out in recent years, the pathways involved in major histocompatibility complex (MHC) class II-restricted antigen processing have yet to be clearly defined. Experimental evidence points to the central role of the endocytic pathway during antigen proteolysis and further MHC-II and antigenic peptide encounters.[1] A specific compartment or several of them have been implicated in antigen processing and interaction with MHC-II molecules.

It has been shown that unfolding of the native protein is sufficient to bypass the need for proteolysis.[2] More recently, research has indicated a role for thiol in stimulating the rate of lysosomal degradation of disulphide-rich proteins. The existence of a lysosomal transport system that delivers cysteine from the cytoplasm to this compartment was first postulated by Lloyd[3] and recently confirmed by others.[4] Several studies showed the importance of lysosomes in the biochemical processing of defined protein antigens – beef insulin A, hen egg lysozyme (HEL) and bovine ribonuclease (RNAse) – and also supported the hypothesis that disulphide reduction and low pH can suffice for partially unfolded or structurally destabilized proteins to bind MHC-II molecules.[5–7]

Proteolysis and the generation of reactive oxygen intermediates generation are biochemically linked processes. This was deduced from the demonstration that oxidatively modified proteins are the preferred substrates for proteolytic degradation (for reviews see[8,9]) and protease inhibitors antagonize the burst of oxygen consumption as well as the resultant production of O_2^- and H_2O_2.[10,11] This observation correlates with the fact that intact cells exposed to oxidants exhibit increased protein turnover rates.[8,9,12,13] However, the precise mechanism of proteolytic processing of antigens is not fully understood. For example, several lysosomal proteinases, particularly the cathepsin D, have been implicated in antigen processing[1,6] but surprisingly, recent experiments with mice genetically deficient for either cathepsin B or cathepsin D show normal antigen presentation.[1,14]

In this light it seemed of interest to examine the participation of oxidative mechanisms in antigen processing *in vitro* by direct and indirect experimental approaches. In the present study, we considered two independent strategies by which to focus on this question: (i) blocking reactive oxygen intermediates by using defined inhibitors or scavengers; and (ii) *in vitro* oxidation of defined protein antigens followed by tests of these oxidized proteins for specific T-cell recognition using live and fixed macrophages (MØ) as antigen-presenting cells (APC).

Received 21 May 1998; revised 16 July 1998; accepted 16 July 1998.

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MATERIALS AND METHODS

Mice

Five- to six-week-old female C57BL/6 and CBA/J mice, originally obtained from Jackson Laboratory (Bar Harbor, ME),

were bred in our facilities. They were maintained under specific pathogen-free conditions in our facilities housed under laminar flow in microisolator cages and given sterile food and water *ad libitum*. Sentinel mice were checked weekly for murine viruses (Immunocomb, Charles River, Wilmington, MA) and percentages of IA-positive peritoneal exudate cells.

General reagents

$NaIO_4$, NaOCl, H_2O_2, phorbol 12-myristate 13-acetate (PMA), bovine superoxide dismutase (EC 1.15.1.1; SOD), horse cytochrome c, horseradish peroxidase (EC 1.11.1.7), β-nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSSG), ferricytochrome C, phenol red, sulphanilamide, naphthlylethylene diamine dihydrochloride and phosphoric acid were purchased from Sigma Chemical Co. (St. Louis, MO). $Na_2S_2O_3$, formic acid and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). [^{3}H]thymidine was from Amersham Iberica S.A. (Spain). Murine recombinant interferon-γ (IFN-γ) was obtained from Stratagene Cloning Systems (La Jolla, CA). All culture assays were done in RPMI-1640 (R0 medium) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 μg/ml gentamicin sulphate (R10 medium). All media and reagents used were confirmed to be endotoxin free (<0·01 ng/ml) by chromogenic limulus amebocyte lysate (LAL) microassay from Whittaker M. A. Bioproducts (Walkersville, MD).

Radical scavengers and other inhibitors

D-Mannitol, 2-mercaptoethylamine (cysteamine), 1,3-dimethylurea (DMU), 1,3-dimethyl-2-thiourea (DMTU), 3,3′,4′,5,7-pentahydroxyflavone (quercetin), [2R-(2a,6aα,12aα)]-1,2,12,12a-tetrahydro-8,9-dimethoxy-2-(1-methylethenyl)-[1]benzopyrano-[3,4-b]furo[2,3-h][1]benzopyran-6(6aH)-one (rotenone) and antimycins A_1–A_4 were from Sigma. Hydrazinecarboximidamide hemisulphate (aminoguanidine) was from Calbiochem (San Diego, CA). Bis[ethyl(2R,3R)-3-[(*S*)-3-methyl-1-[4-(2,3,4-trimethoxyphenyl-methyl) piperazin-1-yl-carbonyl-]butylcarbamoyl]oxirane-2-carboxylate]sulphate (NCO-700) was from Kamiya Biomedical Co. (Thousand Oaks, CA). Quercetin was dissolved in alkaline solution and titrated to a pH 7·2 maintained under nitrogen to avoid auto-oxidation. DMU, DMTU and cysteamine were freely soluble in medium. Rotenone and antimycins were dissolved in ethanol. Mannitol was dissolved in hot phosphate-buffered saline (PBS).

Protein antigens and chemical modifications

Hen egg lysozyme (HEL), bovine α-lactalbumin (BLA), and ovalbumin (OVA) were from Sigma and purified by fast performance liquid chromatography (FPLC) using a mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden). For some experiments, proteins were carboxymethylated as follows. Approximately 10 mg of each purified protein were added to a solution containing 8 M urea, 1 M acetic acid buffered with 1 M Tris base containing 50 mM ethylene diamine tetra-acetic acid (EDTA). After 1 hr incubation at room temperature, the solution was diluted with H_2O and adjusted to pH 5 with acetic acid. Gaseous N_2 was bubbled through this for 15 min with constant stirring and 100 μl of 2-mercaptoethanol were added. The mixture was incubated at 40° for 60 min and then dialysed with PBS at 4°.

Oxidation of proteins with performic acid was according to previously published protocols.[15] Briefly, performic acid, prepared by adding 0·005 ml of 30% H_2O_2 to a solution of 20 mg of the purified protein in 0·2 ml of formic acid, after 30 min at room temperature had 10 ml of distilled water added before solvent removal by lyophilization. Protein chlorination was performed exactly as previously described:[16] proteins at a concentration of 2 mg/ml in 0·05 M phosphate buffer (pH 7·6) were dialysed for 72 hr at 4° against 0·05 M phosphate buffer (pH 7·6) containing NaOCl. HOCl concentrations for appropriate protein oxidation and subsequent optimal presentation by fixed MØ were: 0·02–0·2 μmol/mg protein for OVA and 1–5 μmol/mg protein for both HEL and BLA. The ratio of external to internal compartment volumes was 100:1. Protein chloramines and excess HOCl was removed by equimolar concentration of $Na_2S_2O_3$. The latter and its oxidation products were removed by further 24 hr dialysis against the same buffer. Native protein controls were treated similarly to the chlorinated proteins except for the NaOCl addition.

Measurement of ROI and RNI in MØ

Production of O_2^- was measured by the SOD-inhibitable reduction of ferricytochrome C as described.[17] Results are expressed as nmol O_2^- produced per mg of protein. H_2O_2 production was measured by a technique based on the HRPO-dependent conversion of phenol red by H_2O_2 into a compound with increased absorbance at 600 nm.[17] Results are expressed as nmol H_2O_2 produced per mg of protein. The generation of nitrite (NO_2^-) by MØ was determined by the microplate method of Ding *et al.*[18] Results of unknown culture fluids were expressed as nmol of NO_2^- produced per well; which was derived from a sodium nitrite standard curve.

Antigen-presenting cells, T-cell hybridomas and class II restricted antigen-presentation assay

Concanavalin A (ConA)- or *Listeria monocytogenes* (LM)-elicited peritoneal MØ (in these cases MØ were 60–95% IA^+) were obtained as previously described.[19,20] They were cultured at 37° in 96-well tissue culture trays (Costar Cambridge, MA). Paraformaldehyde-fixed and live MØ were used as APC. The following T-cell hybridomas were used: 3A9 and 2A11 (specific for HEL (52–61) IA^k).[21] D6.37.3.20 (specific for an IA^k-restricted unidentified BLA peptide),[22] and DOBW (specific for OVA (323–339) IA^b/IA^d).[23] Antigen presentation was performed as previously described.[24]

RESULTS

Effect of different ROI and RNI inhibitors on MHC class II-restricted presentation

Selective inhibitors are extremely useful probes for characterizing target molecules or defining function, particularly when the targets are critical in intracellular networks. The effects of several putative scavengers on both ROI and RNI generation as well as on HEL presentation by MØ to 3A9 cells were first titrated over a large range of concentrations (Fig. 1) and IC_{50} calculated in each case. The information obtained was: (i) compounds that do not (DMU) or only moderately (quercetin) modify antigen presentation and generally do not (DMU) or only slightly (quercetin) depress ROI formation; and (ii) chemicals that clearly depress both ROI generation and

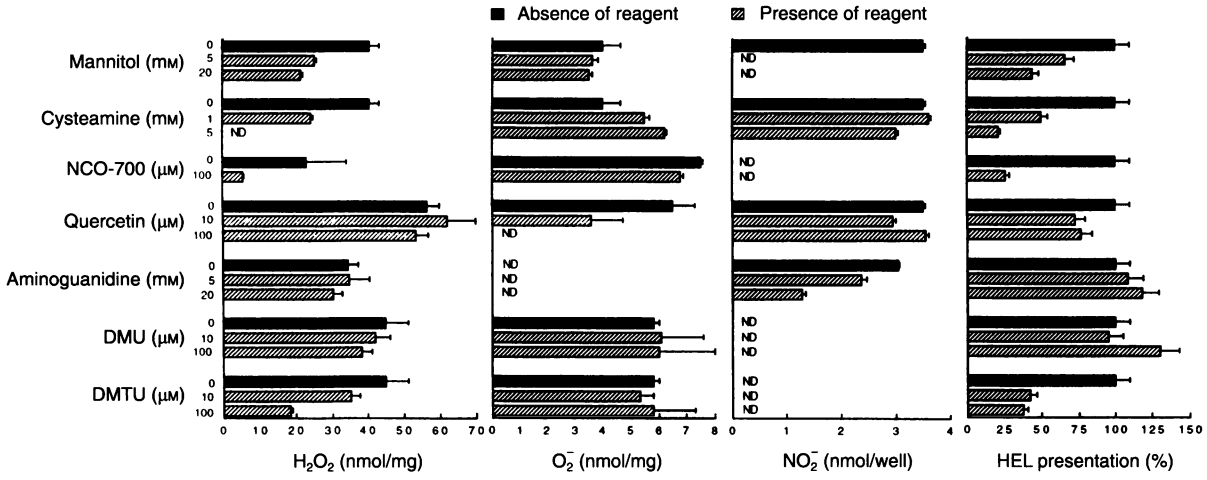

Figure 1. Effect of scavengers and inhibitors on the generation of both oxygen and nitrogen intermediates as well as on antigen presentation. Parallel sets of IA^k-positive peritoneal MØ were assayed for H_2O_2, O_2^- and NO_2^- production as well as for presentation of HEL, in the absence or presence of the indicated reagents, as described in the Materials and Methods. Results are expressed as the mean ± SD of triplicate determinations. Values of HEL presentation are expressed as the percentage of response with respect to the untreated control (100%). DMU, 1,3-dimethylurea; DMTU, 1,3-dimethyl-2-thiourea, ND, not done.

antigen presentation in a dose response manner (e.g. mannitol, cysteamine, NCO-700, and DMTU). It is important to mention that the MØ viability was unaffected by these scavengers, neither at the concentrations tested, nor over the experimental time course. However, experiments with scavengers were only considered valid if the degree of inhibition produced correlated with the rate constants for scavenger reaction with ·OH. The IC_{50} for O_2^- and H_2O_2 inhibition were 52·5 mM and 14 mM for mannitol and 536 μM and 66 μM for NCO-700, respectively. Cysteamine and DMTU only inhibited H_2O_2, at IC_{50} of 2·7 mM and 55 μM, respectively. These specific scavengers impaired the presentation of native protein antigens (mannitol, IC_{50} = 750 μM; cysteamine, IC_{50} = 3·1 mM; DMTU, IC_{50} = 8·72 μM and NCO-700, IC_{50} = 67·7 μM). These data indicate a better correlation of antigen presentation impairment with H_2O_2 release inhibition than with O_2^- inhibition (4–10 times higher inhibitor concentration required to inhibit O_2^- production). In some cases, however, products of the cysteamine reaction with oxidants may themselves exert deleterious biological effects.[25] Natural bioflavinoids (e.g. chrysin, apigenin, kaempferol, morin and myricetin) do not inhibit either antigen presentation or ROI generation or only marginally (data not shown). Although flavinoids scavenge hydroxyl, superoxide and peroxyl radicals, this proposal may have been overinterpretated (see 26 for a review on this issue). Only quercetin was able to inhibit antigen presentation. However, it is known that the ATP-binding site of PI-3-kinase is competitively inhibited by quercetin and by LY294002, a quercetin-derived synthetic analogue (IC_{50} = 3·8 μM and 1·4 μM, respectively).[27,28] Interestingly, LY294002 and wortmannin, another specific inhibitor of PI-3-kinase and PLD activation, also block antigen processing in MØ, but not in B cells or dendritic cells (16 and unpublished observations).

Consequently, only the following were selected for further experiments: mannitol (an ·OH scavenger,[29]), DMTU (which reacts with H_2O_2 and HOCl,[30,31]) and NCO-700 (an epoxysuccinic acid derivative which inhibits oxidant production by activated neutrophils and can scavenge reactive oxygen species in both NaOCl and hypoxanthine-XOD systems,[32]). Finally, it is also of interest to point out the existence of a hierarchy in antigen presentation inhibition that correspond exactly to the order of impairment of H_2O_2 production (DMTU > NCO-700 > mannitol).

It is generally assumed that RNI are also triggered in addition to the ROI generated with the respiratory burst. Aminoguanidine, a inducible nitric oxide-synthase (iNO-synthase) inhibitor[33] only inhibited RNI generation as expected (IC_{50} = 14 mM) and caused no effect on antigen presentation (Fig. 1). This inhibitor did actually slightly stimulate HEL presentation to 3A9 cells by unknown mechanisms.

ROI scavengers do not inhibit the MHC class II-restricted presentation of antigens that need processing but have their disulphide bonds reduced

As described above, processing of native protein antigens by MØ is blocked by select radical scavengers. Whether these inhibitors affected processing of a protein antigen when its disulphide bridges are reduced was therefore studied. For this experiment a T-cell hybridoma, 2A11[21] was used, which does not respond to CM-HEL when *p*-formaldehyde-fixed MØ or chloroquine-treated MØ are used as APC, indicating that this carboxymethylated protein requires intracellular processing despite being unfolded.[21] The experimental procedure was to test both native and carboxymethylated antigen for processing using untreated MØ and MØ treated with ROI inhibitors. As expected (Fig. 2), mannitol, DMTU and NCO-700 do not inhibit processing by MØ of CM-HEL (lower panels) in contrast to their blocking effect on the processing of native HEL (upper panels). The fine specificity of the chemical target is also illustrated: DMTU inhibited antigen processing whereas a chemically related compound, DMU, was ineffective. This dose dependent inhibition was reproduced with a BLA specific T-cell hybridoma (D6.37.3.20 cells), which behaves as 2A11 (data not shown).

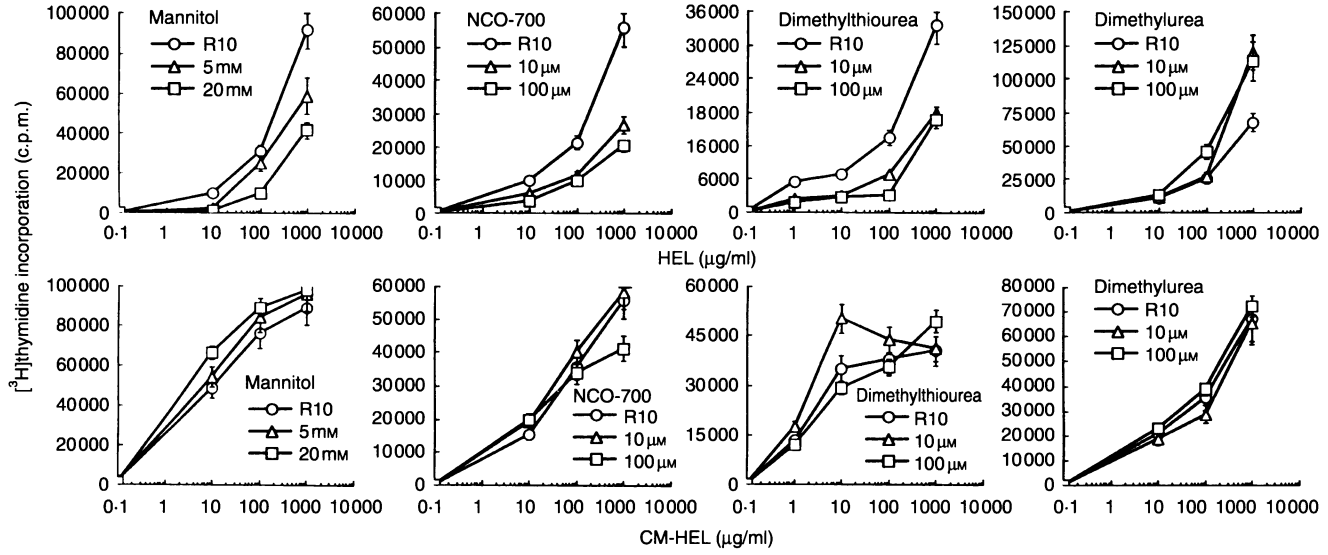

Figure 2. ROI scavengers do not inhibit presentation of protein antigens that need processing but have their disulphide bonds reduced. IA^k-positive peritoneal MØ were cultured at 1×10^6 cells/ml with or without (R10) the indicated compounds at 10 and 100 μM (except mannitol which was tested at 5 and 20 mM) for 1 hr, washed, pulsed with different amounts of HEL (upper panels) or CM-HEL (lower panels) concentrations (0–1000 μg/ml) for an additional 1 hr, washed again, fixed with 1% *p*-formaldehyde and tested for presentation to 2A11 hybridoma cells, as described in the Materials and Methods. Results are expressed as the mean ± SD c.p.m. of [^{3}H]thymidine incorporation of triplicate cultures.

Inhibitors of mitochondrial respiration do not inhibit antigen presentation

The mitochondrial respiratory chain produce O_2^- radicals at two sites: flavoprotein NADH dehydrogenase and the ubiquinone-cytochrome b region.[34] Very low concentrations of rotenone and antimycins specifically inhibit O_2^- production at the NADH dehydrogenase and ubiquinone-cytochrome b regions, respectively.[35] We have tested these same drugs, for antigen presentation inhibition. Neither rotenone (up to 10 μM) nor antimycins (up to 1 mM) significantly decreased MHC class II-restricted antigen presentation. A representative experiment is shown in Fig. 3.

Oxidation of protein antigens enhance their immunogenicity

Classically, the ability of phagocytes to kill pathogens has been attributed to a wide variety of agents that are either present in these cells or elaborated during phagocytosis (for review see 36). One of these systems has attracted considerable interest – the myeloperoxidase (MPO)–H_2O_2–Cl^- system.[37] Its toxic reactive product is HOCl.[38] It is, therefore, highly probable that, this chlorinating system could contribute, either alone or in combination with the proteolytic system, to immunogenic peptide cleavage and efficient antigen presentation.

To test this hypothesis defined proteins (HEL, BLA, OVA)

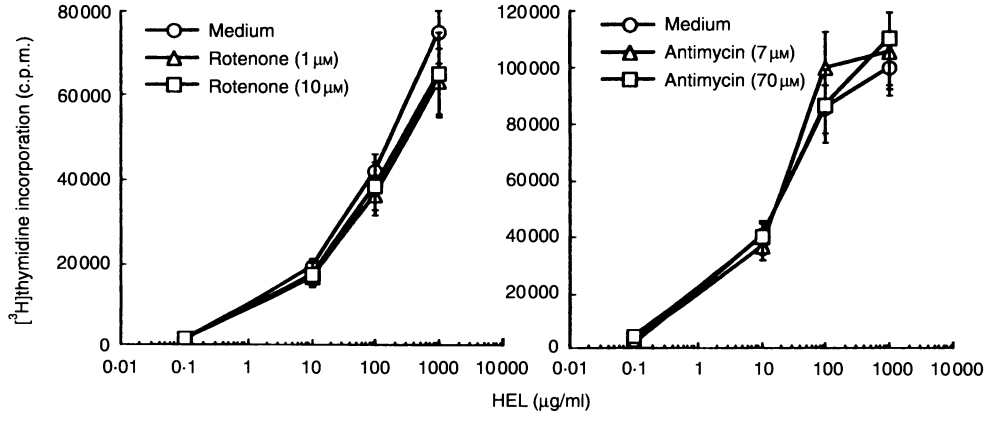

Figure 3. Rotenone and antymicins, inhibitors of the mitochondrial respiration, do not inhibit HEL presentation to 3A9 cells. IA^k-positive peritoneal MØ (1×10^6 cells/ml) were cultured with varying concentrations of rotenone (0–10 μM) and antimycins (0–70 μM) for 1 hr, washed, pulsed with HEL (0–1000 μg/ml) for an additional 1 hr, washed again, fixed and tested for presentation to 3A9 hybridoma cells as described in the Materials and Methods. Results are expressed as the mean ± SD c.p.m. of [^{3}H]thymidine incorporation of triplicate cultures.

were chlorinated and assayed in parallel with native proteins for presentation to their specific T cells using both fixed and live MØ. As is shown in Fig. 4, chlorination enhances HEL presentation by live MØ to specific T-cell hybridomas (left panel). When paraformaldehyde-fixed MØ, which can not process native HEL, were used as presenter cells, activation of T cells was exclusively produced when the protein was optimally chlorinated (right panel). Similar results were obtained when other proteins (BLA, OVA), whether chlorinated or not, were tested for presentation to specific T cells (data not shown).

To extend these observations, additional experiments were performed using a different approach to oxidizing HEL. Consequently, we used the Sanger's method by which it is possible to cleave the disulphide bridges in the protein molecule (conversion of cysteine to cysteic acid residues), by the combined action of H_2O_2 and formic acid.[15] Native or performic-oxidized HEL were added to fixed or unfixed MØ and tested for presentation to 3A9 hybridoma cells. As shown in the left panel of Fig. 5, performic-oxidized HEL was again more efficiently processed by live MØ than native protein and only performic-oxidized HEL was recognized by 3A9 cells when MØ were prefixed with *p*-formaldehyde (right panel). An additional experiment with BLA and specific T cells gave similar results (data not shown).

In conclusion, it is suggested that the oxidation of the native antigen not only improves processing, but also allows direct recognition by the specific T cell in the context of IA molecules on the surface of prefixed APC.

DISCUSSION

Radical scavengers can be used to abolish intracellular redox processes in intact cell preparations or to block a defined molecule whether involved or related. For this purpose we first selected some inhibitors which were generally effective at low concentrations, were non toxic, penetrated intact cells and whose degree of inhibition correlated with antigen presentation impairment. Accordingly, mannitol, DMTU (but not DMU) and NCO-700 impaired the presentation of native protein antigens but were no inhibitory it when these proteins were previously reduced and their free sulphydryl groups alkylated to prevent refolding, forming carboxymethylated proteins. Studies with NCO-700, however, must be interpreted with caution, because the specificity of this scavenger is not absolute.[32] These observations suggest that ·OH play a role in protein unfolding at the level of the reduction of antigen disulphide bonds and that ·OH scavengers are ineffective when carboxymethylated antigen (i.e. unfolded) was added. In addition, our results also suggest that these scavengers are not substantially active on proteolytic cleavage of our antigens.

On the other hand, the observation that antimycins and low concentrations of rotenone do not impair antigen processing was not surprising. It could indicate that these processes are not dependent on mitochondrial oxidation. Our observations coincide with previous reports where it was suggested that both peripheral blood monocytes and alveolar MØ depend on oxidative phosphorylation for their energy supply whereas neutrophils and peritoneal MØ do not.[39,40]

Changes in the structure and function of individual proteins can be determined by exposing purified proteins to radical-generating systems, as well as to enzymatic and non-enzymatic metal-catalysed oxidation reactions (reviewed in 9). Exposure of proteins to ·OH radicals leads to biochemical modifications of some aminoacids and alteration of both secondary and tertiary structure with excellent correlation between increased hydrophobicity and degradation.[11,12] In this paper proteins were oxidized *in vitro* by chlorination[16] or by the combined action of H_2O_2 and formic acid.[15] These procedures were selected because they resemble *in vivo* reactions.

The HOCl or its dissociated form OCl^-, is produced by phagocytes as follows:

$$H_2O_2 + Cl^- \rightarrow OCl^- + H_2O$$

this reaction being catalysed by myeloperoxidase (donor H_2O_2-oxidoreductase).[41] As a strong oxidant, OCl^-, can react with SH and NH_2 groups, thus modifying proteins. This reaction is very fast (e.g. bacteria exposed to HOCl are killed within

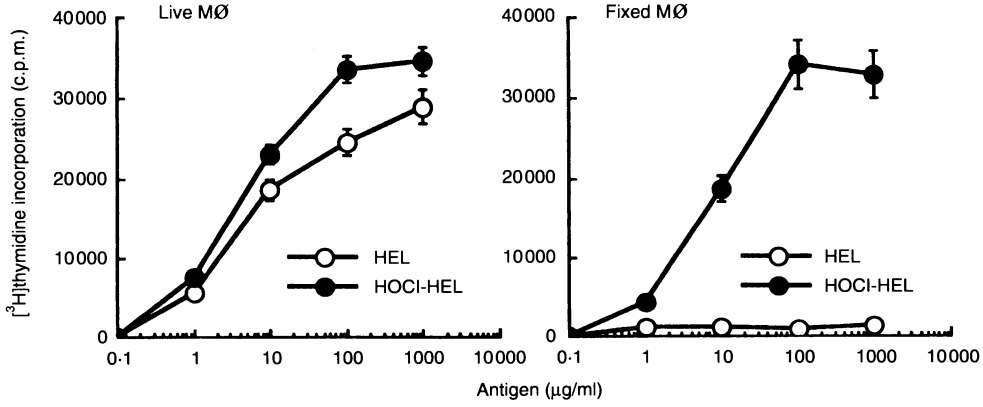

Figure 4. Chlorination of protein antigens enhances their immunogenicity but T-cell activation is dependent of the extent of the oxidation. Left panel: live IA^k-positive peritoneal MØ (1×10^6 cells/ml) were pulsed with increasing concentrations (0–1000 μg/ml) of native or chlorinated HEL (HOCl–HEL; chlorination performed with 0·1 μmol HOCl/mg HEL) for 1 hr, washed, and fixed. Right panel: *p*-formaldehyde-fixed MØ, incubated with the indicated HEL concentrations, chlorinated or not, and washed. MØ sets as described, were tested for presentation to 3A9 hybridoma cells. Results are expressed as the mean ± SD c.p.m. of [^{3}H]thymidine incorporation of triplicate cultures.

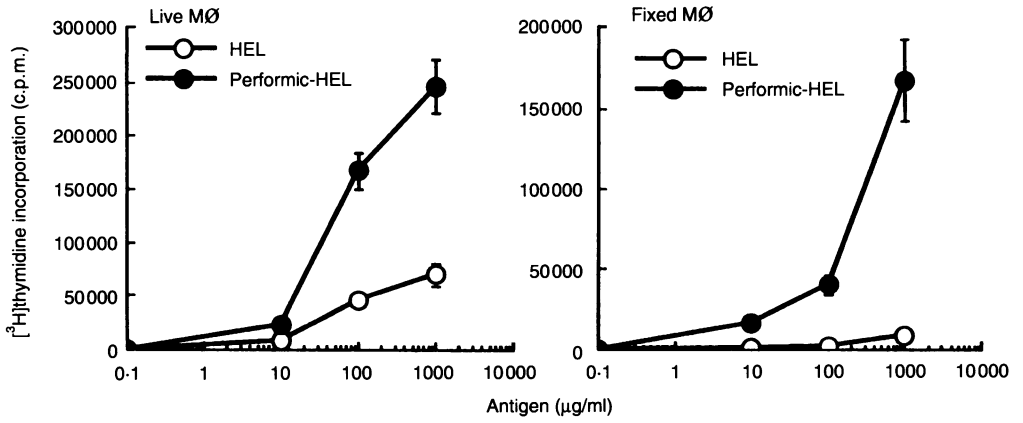

Figure 5. Oxidation of HEL with performic acid increase proteolytic processing and exposes peptide epitopes recognized by 3A9 cells. Approximately 1×10^6 cells/ml live peritoneal MØ (left panel) or *p*-formaldehyde-fixed MØ (right panel), pulsed with increasing concentrations (0–1000 μg/ml) of native HEL or oxidized with performic acid (performic-HEL) for 1 hr and washed (live cells fixed and washed). MØ, then tested for presentation to 3A9 hybridoma cells. Results are expressed as the mean ± SD c.p.m. of [^{3}H]thymidine incorporation of triplicate cultures.

milliseconds following addition of HOCl).[42] Protein sulphydryls are particularly sensitive to oxidation by HOCl. Tryptophane and methionine residues can be oxidized by HOCl at concentrations as low as 10 μM.[43] At least 30–40% of the H_2O_2 produced by stimulated neutrophils is converted into HOCl,[41] hence local HOCl formation may well be in the 100 μM range, a concentration that largely exceeds the amounts used for optimal protein chlorination *in vitro*.[43] It was shown that chlorination of OVA results in its enhanced immunogenic properties.[44] In the present report we have also obtained similar conclusions: chlorination of protein antigens allow protein unfolding and enhances both processing and exposure of immunogenic peptides to specific T cells.

However, several other oxidative reactions take place inside the MØ. Catalase, for example, besides catalytic activity, oxidizes formic acid (an H donor).[45] The hydrated electron is a reducing radical which reacts rapidly with many aminoacids, particularly histidine and oxidized cysteine. The formate radical ion, $\cdot CO_2^-$, formed by the reaction of hydroxyl radicals with formate

$$\cdot OH + HCOO^- \rightarrow \cdot CO_2^- + H_2O$$

is a more selective reducing species than the hydrated electron. At pH 7 this radical reacts with disulphide bridges and other aminoacids:[46]

$$\cdot CO_2^- + RSSR \rightarrow \cdot RSSR^- + CO_2$$

Unlike the hydrated electron, $\cdot CO_2^-$ can only react with accessible disulphide bridges because its size does not permit diffusion into the inner regions of the protein structure. Pulse radiolysis studies, for example, have shown that the disulphide bridges in HEL are more accessible than those in RNAse.[47] This could also help to explain why immunogenic peptide generation following disulphide reduction, with or without *in vitro* enzyme digestion, is easier in HEL than RNAse.[6] We have also demonstrated that performic acid treatment of protein antigens increase both proteolytic processing and exposes immunogenic peptides.

It should be noted that no aminoacid residue presented in the epitopes HEL(52–61) or OVA(323–339) correspond to any of the main targets susceptible to HOCl attack or performic oxidation (Trp and Met or Cys, respectively). In summary, by direct and carefully controlled *in vitro* oxidation of defined protein antigens (HEL, BLA, OVA) these oxidized proteins can be clearly demonstrated not only to be better and faster processed by MØ, but also directly recognized by specific T cells in the context of MHC class II molecules without an intracellular step.

Control of the cellular redox state represents the main regulatory pathways of free radical production. Much direct and indirect evidence showed correlation between redox state and protein degradation mechanisms (reviewed in 8,9). Indirectly, antigen proteolysis can be regulated through the activation of endogenous proteinase inhibitors.[48] It was also reported that after removal or inhibition of antioxidant molecules (GSSG, catalase) intracellular proteolysis is significantly enhanced.[49]

Oxidative processes can occur inside phagolysosomal organelles as well as extracellularly. The experiments described here were not addressed towards whether antigen processing – including potencial redox processes – is performed in a particular intracellular compartment. Nevertheless, it is indeed possible that this can occur inside intracellular vacuoles. It should be taken in account, however, that organelle resistance to oxidative stress is a function of the ability to degrade H_2O_2 into the acidic vacuolar compartment, the lysosomal membrane resistance itself and the amount of redox-active, low molecular weight iron available.[24,50] These parameters vary between both different and similar cells and between lysosomes of individual cells[50] making it difficult to study these processes biochemically. Nevertheless, the results agree with those demonstrating greater accessibility of antigen to proteases following reduction of the disulphide bonds in lysosomes.[51] When all these redox enzymatic systems perform efficiently, other enzyme systems (intracellular proteinases) acting as a secondary antioxidant defence, could take charge of the further removal of oxidatively damaged antigens, which in turn are a source of additional free radicals. This mechanism of disulphide bond cleavage by reduction and/or oxidation, oxidative breakdown of the protein and enzyme proteolytic cleavage,

whether sequential or simultaneous can operate in MØ and other phagocytes acting as APC.

Hypothetically, it is also possible that an immunogenic motif could be exposed *in vivo* by oxidants released at the pericellular microenvironment caused by MØ themselves or other neighbouring cells (e.g. neutrophils, eosinophils), thus accelerating and/or amplifying the specific immune response.[52,53] For example, chemically modified proteins induced higher *in vivo* humoral response than the unmodified protein.[54] Since stimulated neutrophils chlorinate proteins *in vivo*, this group proposed that they could take part in the induction phase of immune response by facilitation of antigen presentation.[52,54]

We can make two final observations. First, Küpffer cells and endothelial cells present antigen somewhat less effectively *in vitro* than a control population of peritoneal MØ.[55] Interestingly, Küpffer cells are deficient in their capacity to release ROI (H_2O_2 and O_2^-) and to dispose intracellular parasites normally *in vitro*, even following IFN-γ exposure.[56] Whether or not these observations are related, was not evaluated. Second, we were able to provide a clear evidence of antigen processing and presentation functions for eosinophils.[57] These cells, therefore, can be regarded not only as phagocytes playing a pivotal role in allergic reactions, but also in specific immune responses as efficient APC. Participation of the powerful respiratory burst generated in these cells compared to MØ in antigen processing remains also to be elucidated.

ACKNOWLEDGMENTS

The two first authors contributed equally to the paper and were supported by the Spanish Ministry of Education and Science and by the 'Marqués de Valdecilla' Foundation, respectively. They and P.L.-M. contributed to immunochemistry and functional assays. E.C.-M. and C.A.-D. made intellectual contributions to the work; F.L.-C. also contributed intellectually, provided funding and was the general advisor. This research was supported by programs DGICYT PB94/1063 and FIS 94/0933. We would also like to thank R. Martínez-Palencia for excellent technical assistence and to I. M. Outschoorn for critical reading of the manuscript.

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